Structure and environmental drivers of target resting stage of phytoplanktonic assemblages in Central Mediterranean Sea

Running page head: Ecology of Mediterranean phytoplankton resting stage assemblages

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Abstract

Phytoplankton species can produce resting stages that persist in the sediments even for long periods accomplishing important ecological functions. With the aim of investigating the assemblage structure in relation to environmental drivers and human pressures, resting stages of diatoms and dinoflagellates including HAB target taxa were analyzed in surface sediments of three Mediterranean regional areas. Abundance of resting stages were determined by molecular qPCR. Multivariate data analysis confirmed that the abundance of resting stage

assemblages seemed related to depth. Regional differences in the composition of the resting stage assemblages were evident and environmental drivers were correlated with those regional differences. Three main groups of samples were defined according to SST and depth thresholds. Samples from Northern and Central Adriatic Sea (average SST < 18°C) form the richest assemblages, both in terms of abundance and species richness, while deep samples from all the other basins (depth > 368 m) are the poorer and less diverse than those from shallower sites. Resting stage taxa contributed differently in the three groups. Diatom spores and dinoflagellate cysts were the most abundant taxa, but *Alexandrium minutum* cysts and *Ditylum brightwellii* spores also accounted for a large share of the overall inter-group distance. The structure of resting stage assemblages can be regarded as the time- and space-integrated response of a subset of phytoplankton species to the environmental conditions including the physical oceanographic dynamics that favored or prevented sedimentation of resting stages.

Key words: resting stages, assemblages, structure, sea surface temperature, depth, Mediterranean Sea, phytoplankton.

1. INTRODUCTION

Water column and sediments represent two most different, but linked compartments defined by specific environmental conditions where microbial assemblage structure achieves its functions (Taylor 1987, Calbet 2008).

Several microalgal taxa, including HAB (harmful algal bloom) species, produce resting stages that deposit onto the sea bottom forming the seed banks or assemblages which play a crucial role in maintaining microbial populations in future environments (Lennon & Jones 2011). Resting stages may remain quiescent in sediments for decades or years facing environmental condition variability and potentially constituting the inoculum for future assemblages in the water column (Kremp et al. 2009, Lundholm et al. 2011, Montresor et al. 2013).

The two major components of phytoplankton assemblage in temperate areas are dinoflagellates and diatoms. They are able to produce resting stages characterized by different morphology and eco-physiology. In particular, the diatom *Pseudo-nitzschia* spp. are generally known to do not produce resting spores, but, it is also documented that stages of non-viable dormant stages, resting cells or quiescent phase were retrieved in the sediments (Amato et al. 2005, Lundholm et al. 2010, Zhang et al. 2010, Orlova & Morozova 2009, Lelong et al. 2012). However, limited information is available on the benthic resting spores produced by *Pseudo-nitzschia* spp. (Hubbard et al. 2014).

The ecological role of the resting stages in coastal sediments may play a significant function for maintaining the primary production under seasonal cycling or long-term periods. Dinoflagellate and diatom resting stages may accomplish primarily in short term (seasonal) or long term (decades) survival, even if resting stage formation and deposition are long time processes in order to act in response to survival strategies (Radzikowski 2013). But, the production of asexual temporary resting stages, as dinoflagellate temporary cysts and diatom resting cells, may have a function for short-term survival stages than the resting stages (Kuwata et al. 1993, Figueroa & Bravo 2005). Under long term perspective, resting stages play an important role of resilience and recovery of the phytoplankton assemblages from abrupt environmental changes through the high genetic variability of seed banks (Kremp et

al. 2016). Furthermore, phytoplankton seed banks can also provide an important marine ecosystem service in terms of carbon cycling in transfer of carbon to the deep bottom as carbon sinks, and of carbon storage in coastal sediments (Ellegaard & Ribeiro 2018). Resting stages can be also passive dispersed by transport on long-distance via currents, winds, mediated by animals and humans. This allows to several phytoplankton species to outspread their distribution and colonization of new areas. In particular, for harmful species, which are associated to toxin production and bloom formation with consequent water quality degradation, this is an actual increasing trend that is often linked to aquaculture activities, ballast waters and plastic dispersion (Bolch & de Salas 2007, Perini et al. 2019, Casabianca et al. 2019).

Environmental variables and hydrodynamic conditions of water column can influence the resting stage assemblages in term of species composition and abundance, as well as the resting stage dynamics by coupling the bentho-pelagic components (Ribeiro & Amorim 2008, Bringué et al. 2018). The environmental conditions can act primarily on the vegetative stages of many phytoplankton species in the water column. Nutrient availability, sea surface temperature, hydrodynamic conditions, irradiance and seasonal period are the main factors affecting resting stage assemblages. However, the time-lag and spatial uncertainty existing between the encystment processes from pelagic compartment and records in sediments associated to the species-specific ecophysiology makes the understanding processes of the resting stage pattern difficult (Harland et al. 2004, Anderson & Rengefors 2006).

The Mediterranean Sea is an oligotrophic basin highly differentiated in sub-regions based on the hydrodynamic and climatological regimes, and human coastal pressures (Reygondeau et al. 2017, Ayata et al. 2018). Those differences imply that trophic conditions and productivity are different, as well as the structure and dynamics of the phytoplankton assemblages in the water column. It is plausible to assume that also the resting stage assemblage composition

can retain variability traits coupled to the pelagic component (McQuoid & Godhe 2004). Areas of high productivity are mainly restricted to water close to major freshwater inputs of rivers (D'Ortenzio & Ribera d'Alcalà, 2009, Ludwig et al. 2009); but, coastal ecosystems impacted by anthropogenic nutrient input can be influenced by local hydrographic variability and conditions, which can trigger site-specific effects and patterns. Further, human activities are potent new drivers that significantly affect the functioning of coastal and off-shore marine ecosystems both in the pelagic and benthic compartments (i.e. net trawling by fishery, dredging, translocation, eutrophication, ballast water discharge and mariculture practices) (Hallegraeff 2010, Huertas et al. 2011, Penna et al. 2017). Consequently, the study of phytoplankton resting stage structure and its correlation with environmental variables and human pressures over time and space is fundamental in order to clarify the benthic-pelagic coupling and to verify the potential anthropogenic perturbations in the marine sediments, especially in coastal and open areas poor or seldom investigated as the Mediterranean Sea.

The present study examined the resting stage assemblage structure from three sub-regions in the Mediterranean Sea. Through several surface sediment samples collected at various depths between November 2015 and August 2017, we estimated the abundance of main taxonomical groups of dinoflagellate and diatom, and one Raphidophyceae resting stages, including also HAB taxa, using molecular qPCR (quantitative polymerase chain reaction) assay in relation to several environmental variables and human impact. Statistical analyses provided clear insights into the relationships between resting stage assemblages and drivers in the marine ecosystems surrounding Mediterranean coastlines.

2. MATERIALS AND METHODS

2.1. Sediment sampling

A total of 94 surface sediments were collected at several sampling stations in the marine sub-regions of the Mediterranean Sea, such as Adriatic, Ionian and Tyrrhenian Sea (Table S1) during different cruises operated by CNR (Consiglio Nazionale delle Ricerche) - CoNISMa (Consorzio Nazionale Interuniversitario per le Scienze del Mare) in 2015-2017. All samples were collected at coastal and off-shore areas, while accumulation sites, such as harbours or bays, were not sampled. Sediment samples were collected by means of a gravity Van Veen grab sampler operated on board of Minerva Uno vessel owned by CNR. Surface samples were harvested at depths ranging from 1 to 1018 m. Three samples were collected per each sampling station. The surface portion (1 cm) of each sample was placed in sterile tubes using sterile spatula and kept in the dark at +4°C till processed.

2.2. Microalgal cultures

Target species of *Chaetoceros socialis* CBA22, *Ditylum brightwellii* CBA2, *Skeletonema marinoi* CBA4, *Thalassiosira* sp. CBA3, *Scrippsiella trochoidea* CBA3, were isolated by micropipetting from net surface seawater samples collected at 3000 m off the NW Adriatic coast at Foglia (43°56.85′N, 12°56.10′E) and Metauro (43°50.65′N, 13°03.83′E) stations. Light microscopy observation and isolation of living cells were carried out using an Axiovert 40 CFL, Zeiss at 200x and 400x magnifications. Strains of *A. tamarense* VGO1042, *Gymnodinium impudicum* GY6V, *Heterocapsa triquetra* RCC4813 and *Heterosigma akashiwo* HA2V were obtained from algal collection of Instituto Español de Oceanografía (IEO, Spain). Bacillariophyceae monoclonal cultures were maintained in f/2 medium (Guillard 1975) at 16 ± 1 °C, while various Dinophyceae and Raphidophyceae strains were maintained in different media (Table S2) at 23 ± 1 °C. Light was provided at an irradiance of 100 μE m^{-2} s^{-1} on a standard 14:10 h light/dark cycle. Microalgal cultured subsamples containing 1.0 x 10^5 cells were collected between the 6^{th} and 7^{th} day during the exponential

growth phase and centrifuged at 4000 rpm for 15 min. Pellets were stored at −80 °C until molecular analyses.

2.3. Molecular analyses

In order to ensure that only resting stages survived in the sediments, all sediment samples were stored in the dark at 4°C for several weeks and treated to obtain purified phytoplankton resting stage pellets. Briefly, wet subsamples (about 5 g) were suspended in filtered seawater (0.45 μm) and incubated in an ultrasonic bath (Bandelin, Germany) for 2 min at room temperature to separate resting stages from sediment particles. The suspension was then sieved through a steel mesh (Endecotts, UK) using 100 and 20 μm size fractionation, washed with seawater. Resting stages were separated from detrital material by Bolch's (1997) density gradient method, using sodium polytungstate solution (SPT) and a centrifugation step. The interface containing resting stages was placed into a new tube, filled with filtered seawater, and centrifuged twice to recover resting stage material (for details see Perini et al. 2019).

In order to remove the potential DNA debris from dead vegetative cells, resting stage pellets were suspended in 500 μl of distilled water, incubated at +75 °C for 10 min and centrifuged at 4000 rpm for 7 min following the protocol developed by Kim et al. (2016). Genomic DNA extraction from both cultured microalgal cell and resting stage pellets was performed using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Finally, DNA was quantified using a Qubit fluorometer with a Quant-iT dsDNA HS assay Kit (Invitrogen, Carlsbad, CA, USA) and stored at −20 °C until molecular analyses.

In the present study, the sediment samples were analysed for the quantification of a total of 17 diatom and dinoflagellate taxa by molecular qPCR assay (Table S3). Seven new taxon-specific primers for *C. socialis*, *D. brightwellii*, *Skeletonema* spp, *Thalassiosira* spp., *Gymnodinium* spp., *H. triquetra* and *S. trochoidea* species complex were designed on LSU

(large subunit) or 5.8S-ITS (5.8S subunit and internal transcribed spacers ITS1 and ITS2) rDNA gene sequences available from GenBank using Primer-BLAST (Ye et al. 2012). Multiple sequence alignments were carried out using CLUSTALX2 v. 2.0 (Larkin et al. 2007). The specificity of the new primers was tested *in silico* using BLAST (Basic Local Alignment Search Tool) and by qPCR with genomic DNA from non-target species (Table S2). Moreover, primers for the quantification of Dinophyceae and Bacillariophyceae were used as described in Casabianca et al. (2019). The qPCR quantifications of other 8 phytoplankton taxa as *A. minutum*, *A. pacificum, G. spinifera*, *L. polyedrum*, *P. reticulatum* (Perini et al. 2019), and *A. tamarense/A. mediterraneum*, *Pseudo-nitzschia* spp. (Penna et al. 2007), and *H. akashiwo* (Yuan et al. 2015) were also performed. The qPCR reactions were done in a final volume of 25 µl using the Hot-Rescue Real Time PCR Kit-SG (Diatheva, Fano, Italy), 0.5 U of Hot-Rescue Taq DNA polymerase and 1 µl undiluted and 1:10 diluted DNA template. Primer and $MgCl_2$ concentrations, melting temperatures, amplicon sizes and rDNA target region are shown in Table S3. The thermal cycling conditions consisted of 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All amplification reactions were carried out in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Standard curves were included in each PCR reaction. All samples were run with three biological replicates, each of which was run with two technical replicates.

Standard curves for the seven new primer sets were constructed using a 6-point tenfold dilution series of purified rDNA PCR products (from 2 to 1.0×10^6 copies) generated from DNA of the target species as described in Perini et al. (2019). The *A. tamarense* CNRATAA1 plasmid was used for the *A. tamarense/A. mediterraneum* standard curve generation. Standard curves for quantification of the remaining target taxa were previously described (Penna et al. 2013, Yuan et al. 2015, Perini et al. 2019). In particular, for the quantification of Dinophyceae and Bacillariophyceae, the cellular standard curves were obtained by mixing

purified DNAs from 20 different monoclonal cultures of microalgae belonging to the two classes. Based on the assumption that the number of copies of SSU rDNA is proportional to the biomass of the species, a variety of class size of dinoflagellates and diatoms with different range of copy number were used (Godhe et al., 2008, Penna & Galluzzi, 2013, Perini et al. 2019, Gong & Marchetti 2019). In the present study, in order to reduce the potential rDNA copy number variability, the cellular standard curves were constructed using a mix of cultured strain cells of dinoflagellates and diatoms (Casabianca et al. 2019). By interpolating the Ct (threshold cycle) determined experimentally on standard curves, the copy number of both cultured strains and purified resting stages from sediment samples were obtained. Multiplication by the dilution factor allowed calculation of the total number of copies g^{-1} of dry weight (d.w.) of sediment sample. This value was then divided by the number of copies $cell^{-1}$ obtained from cultured strains to obtain the resting stages number g^{-1} of d.w. Acquisition of qPCR data and subsequent analyses were carried out using StepOne software v. 2.3 (Applied Biosystems). A dissociation curve was generated after each amplification run to check for amplicon specificity and primer dimers. The automatically generated standard curves were accepted when the slope was between 3.59 and 3.32 (90 – 100% efficiency) and the determination coefficient (r^2) was at least 0.99. Amplification efficiency was calculated as $(10^{(-1/\text{slope})} - 1) \times 100$.

Statistical analyses were performed with non-parametric Spearman correlation test using PAST ver. 3.2 (Hammer et al. 2001).

2.4. Cultured strain sequencing

The strains of *Scrippsiella* spp. and *Chaetoceros* spp. obtained in this study were sequenced to confirm the species-specific taxonomical assignment by ITS-5.8S and LSU rDNA sequence alignment. The sequences of ribosomal genes obtained from new cultured isolates

were deposited in the NCBI (National Center for Biotechnology Information) database. The ITS-5.8S region of the rDNA was amplified and sequenced using the universal primer ITSA and ITSB (Adachi et al. 1994). The LSU rDNA was amplified and sequenced using D1R and D2C primers (Scholin et al. 1994) targeting the D1-D2 region. The PCR reaction for the ITS-5.8S rDNA and LSU rDNA was as follows: tubes contained 25 µl of 1X reaction buffer (Hot Start Taq DNA Polymerase 5U/µl, Biotechrabbit GmbH, Germany), 2.5 mM of $MgCl_2$, 0.75X PCR Enhancer, 200 µM of dNTPs, 200 nM or 400 nM of each primer for ITS and LSU, respectively, 1U Taq DNA Polymerase and 0.5–1 ng of DNA template. PCR thermal cycling conditions were the same as reported in Pugliese et al. (2017). All amplified PCR products were purified using the MinElute Gel Extraction Kit (Qiagen), and the products were directly sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Kit v. 1.1 on the ABI 310 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). Standard thermal cycling conditions were used for both templates setting the annealing temperature according to the template (60 °C and 50 °C for ITS and LSU PCR specific primers, respectively). Difficult templates and repeated regions were solved increasing initial denaturation time and modifying thermal cycling condition as follows: 40 cycles of denaturation at 96 °C for 10 s and annealing/extension at 50 °C for 4 min. The *Scrippsiella* spp. and *Chaetoceros* spp. ITS-5.8S and LSU sequences were aligned *in silico* using BLAST database.

2.5. Statistical methods

The resting stage assemblage data set included the abundances of 17 taxa (Table S3) as number of cysts or spores g^{-1} d.w. sediment at 94 sampling stations (Table S1). Moreover, at each sampling station, depth was recorded, whereas the values for other 20 environmental variables were retrieved as the average value in a 1 nm radius from GIS (Geographic

Information System) raster layers with a pixel size of ¼ minute (Table S4). Bathymetric data were obtained from EMODNet Hydrography Portal (2013). Environmental variables were not selected among those available at adequate spatial scale on the basis of potential causal relationships with the structure of the resting stage assemblages, as those relationships are obviously unknown. Moreover, all the selected environmental variables express properties that are steady state or still meaningful when averaged in time. Needless to say, physical properties of the water column or patterns in currents would have been useful from a theoretical viewpoint, but they were not available at our study scale. As for the amount of fishing effort deployed by the fleet of Italian trawlers with length-over-all (LOA) larger than 15 m, it was estimated as total fishing time (in hours) in the year 2016, with respect to a 0.01 degree square grid. The activity of 1170 trawlers were reconstructed from a Vessel Monitoring System (VMS) database provided, within the framework of Data Collection in the Fisheries Sector (https://ec.europa.eu/fisheries/cfp/fishing_rules/data_collection_en), from the National Ministry of Agricultural Food, Forestry, and Tourism Policies. VMS is the most important and reliable remote tracking device used worldwide (Amoroso et al. 2018) to monitor and control fishing activities. Although VMS is mandatory only for large fishing vessels, it was repeatedly demonstrated that it is able to capture the largest portion of pressure and related impacts exerted by the fleets (Russo et al. 2016). VMS data were processed using the VMSbase platform, which implements standard procedures for cleaning, interpolation and identification of fishing set positions (Russo et al. 2014, Eigaard et al. 2017). Other activities (e.g. steaming or in harbor parking) were excluded. All GIS activities were performed under QGIS 2.16.2 (QGIS Development Team, 2015).

Resting stage abundances were log-transformed as $x' = \log_{10}(x+1)$ before any statistical analysis in order to reduce the heterogeneity in the available values, which spanned over more than 4 orders of magnitude, and to normalize as far as possible their distributions.

Redundancy Analysis (RDA) (Rao 1964, Wollenberg 1977) was selected as the best suited ordination technique to explore the overall relationships between resting stage assemblages and environmental and biotic variables. In fact, it aims at restricting the number of linear combinations of explanatory (environmental) variables that are related to some dependent (biotic) variables to some small rank and it can be regarded as a Principal Component Analysis performed on the projections of the latter variables on the space spanned by the former ones. RDA allowed permutation-based tests for independence between environmental and biotic variables and for significance of each canonical axis. RDA and all related tests were performed using the *rda* function in the *vegan* package (Oksanen et al. 2018) under the R 3.51 statistical computing environment (R Core Team 2018). In order to avoid problems related to collinearity, only environmental variables with a Variance Inflation Ratio (VIR) smaller than 10 according to the vir function from the same package were included in the analysis. As RDA can be regarded as an extension of Principal Component Analysis, it operates in a Euclidean space and therefore Euclidean distances were also used in subsequent analyses.

A Multivariate Regression Tree (MRT) (De'ath 2002) was trained using the *mvpart* function of the *mvpart* 1.6.2 package (De'ath 2014). The MRT was used to split the set of resting stages samples into the most homogeneous subsets that can be obtained on the basis of threshold values for selected environmental variables. The MRT was based on Euclidean distances and 5-fold cross-validation was repeated 100 time. The selection of the most parsimonious tree was carried out using the *plotcp* function of the same package. As any other tree-based Machine Learning classification technique, MRTs are not affected by collinearity, and therefore, all the available variables have been included in the training data set.

The role played by each taxon in determining the differences between the groups of samples defined by the MRT was analyzed by means of the Similarity Percentage (SIMPER) on Euclidean distances (Clarke 1993). SIMPER, as well as uni- and bivariate analyses, were performed using PAST (Hammer et al. 2001).

3. RESULTS

3.1. Sequencing analyses and primer specificity

The rDNA gene sequences of *Chaetoceros* spp. and *Scrippsiella* spp. strains were identified based on sequence similarity using BLAST. One strain was identified as *C. socialis* CBA22 (GenBank accession no. MK881016). The three strains CBA1S, CBA2 and CBA3 of *Scrippsiella* were identified as *S. trochoidea* species complex including *S. acuminata* (GenBank accession no. MK881019), *S. erinaceus* (GenBank accession no. MK881020), *S. trochoidea* var. *aciculifera* (GenBank accession no. MK881021) due to the high level of similarity among ribosomal DNA sequences (100 – 99% of similarity). Therefore, in the present study, *S. trochoidea* species complex was investigated including other potentially closely related species (see also Gottschling et al. 2005, Lee et al. 2018).

The species-specificity of seven new primers designed to target *C. socialis*, *D. brightwellii*, *Skeletonema* spp., *Thalassiosira* spp., *Gymnodinium* spp., *H. triquetra* and *S. trochoidea* species complex was tested *in silico* using BLAST. The results indicated that primers were highly specific. Primer species-specificity was also assayed using DNAs of other phytoplankton species (Table S2). Negative amplifications were obtained confirming the species-specificity of the primers (data not shown).

3.2. Standard curves and abundance of resting stages in sediment samples

Standard curves used to calculate the number of copies in each reaction (Table S5) showed a PCR efficiency from 90 to 100%, a linear relationship over a range of 6 orders of magnitude ($r^2 = 0.99$) and a quantification limit of two copies per PCR reaction. The reproducibility of qPCR assay was analyzed both, as CV_{Ct} (coefficient of variation of cycle threshold) and CV_{Cn} (coefficient of variation of copy number). The CV_{Ct} mean inter-assay variability ranged from 0.5 to 1.4% (1.0×10^6 - 2 copies), while, the CV_{Cn} mean values ranged from 9.9% to 25.5% (1.0×10^6 - 2 copies).

Accurate resting stage quantification of Dinophyceae and Bacillariophyceae and other target phytoplankton taxa was performed by qPCR assay in surface sediment samples. The maximum abundance of dinoflagellate cysts (9878 ± 467 cysts g^{-1} d.w.) was observed at 22 m of depth at MS16_III_147 sampling station, a sampling site about 4 miles south of the Po Delta (Adriatic Sea) during the third campaign of the 2016. The maximum diatom spore abundance (34329 ± 4308 spores g^{-1} d.w.) was found at 41 m of depth at MS17_I_111_C station in Tyrrhenian Sea during the first campaign of the 2017. The lowest values of both taxa, were found at MS16_II_77, Ionian Sea, in the second campaign of the 2016 and at MS17_II_035_C R1, Strait of Sicily, in the second campaign of the 2017 at 930 and 418 m of depth, for Dinophyceae and Bacillariophyceae, respectively. Moreover, no diatom spores were found in three samples collected in the Ionian Sea (at 415, 766 and 930 m depth) and in three samples from Tyrrhenian Sea (at 440, 446 and 495 m depth) (Fig. 1). The abundance of resting stages decreased with depth increasing based on significant negative correlation obtained between depth and the two class' abundance ($n = 94$, Spearman's $rs = -0.73$, $p << 0.001$ and Spearman's $rs = -0.60$, $p << 0.001$ for Dinophyceae and Bacillariophyceae resting stages, respectively). Further, higher resting stages abundance of one class was directly linked to an increased abundance of the other (Fig. 2) based on significant positive correlation ($n = 94$, Spearman's $rs = 0.86$, $p << 0.001$) found between Dinophyceae and

Bacillariophyceae abundance. Further, in the Adriatic Sea sediments, the diatom *D. brightwellii* resting stages were the most abundant with 4911 ± 475 spores g^{-1} d.w. at 33 m depth at MS16_III_165 followed by the highest cyst number of *A. minutum* (1032 ± 77 cysts g^{-1} d.w.) at 35 m depth MS15_55 station (Fig. 3a). In the Ionian Sea, the dinoflagellate *Gymnodinium* spp. cysts were the most abundant (174 ± 14 cysts g^{-1} d.w.) at 188 m depth at MS_152 station followed by the diatom *C. socialis* spore abundance (43 ± 5 spore g^{-1} d.w.) at 130 m depth at MS17_II_041_CR1 (Fig. 3b). Finally, in the Tyrrhenian Sea, the diatom *Pseudo-nitzschia* spp. presumably non-viable dormant stages were the most abundant with the presence of 297 ± 11 spores g^{-1} d.w. at 41 m depth at MS17_I_111_C followed by *C. socialis* spores (149 ± 11 spore g^{-1} d.w.) at 127 m at MS17_I_84_CR1 (Fig. 3c). Among the toxic producing taxa, *Pseudo-nitzschia* spp. non-viable dormant stages and *L. polyedrum* resting stages were found the most frequent in the range of 77-76% of presence in the 94 sediment samples analyzed. Indeed, *A. minutum* and *P. reticulatum* were retrieved in the highest abundance in Adriatic Sea both at 35 m with 1032 ± 77 and 796 ± 87 cysts g^{-1} d.w., respectively. The Raphidophyceae *H. akashiwo* was found in a single Tyrrhenian Sea sediment sample at 127 m depth at MS17_I_84_CR1, with 8 ± 1 cysts g^{-1} d.w. (data not shown).

3.3. Resting stage assemblage analysis

The resting stage assemblage included a very heterogeneous subset of the taxa that can be found in the water column throughout the seasonal successional cycle. Taxa in this subset differed by 3-4 orders of magnitude in their abundances.

While relationships between sampling depth and the abundance of dinoflagellate cysts and diatom spores had been already detected, an overall dependence of the resting stage assemblage on the environmental variables was also detected. A Redundancy Analysis

(RDA) was performed, taking into account resting stage abundances of 17 taxa and 9 environmental variables out of the available 21 (see short names with an asterisk in Table S4, selected among). In order to avoid problems related to strong collinearity, environmental variables with a VIF larger than 10 were filtered out and excluded from the RDA, then the null hypothesis of independence between biotic and abiotic data subsets was rejected on the basis of the ANOVA-like permutation test ($p = 0.001$ with 999 permutations) associated to the RDA. According to the same procedure, only the first two canonical axes were found to be significant ($p = 0.001$ and $p=0.014$, respectively, with 999 permutations), but they accounted for a very large share of variance, i.e. 49.88% (RDA1 44.27% and RDA2 5.59%) (Fig. S1). The RDA allowed visualizing the differences in composition of resting stage assemblages in the three subregional Seas, i.e. Adriatic, Ionian and Tyrrhenian Seas and their association with resting stages taxa and environmental variables. All the resting stages were more frequent in the Adriatic Sea, whose resting stages assemblages were also found to be more abundant. Fishing pressure (fishing in the ordination) was higher in that region than in the others, which were associated to higher values for all the remaining variables, with the only exceptions of seafloor temperature (bottemp, higher because of the limited depth of the Adriatic Sea) and human impact (i.e. impact, as defined by Halpern et al., 2008) that seems independent of differences between basins. While the RDA ordination seemed consistent with the spatial distribution of resting stages, abundances of resting stages were not homogeneous among Adriatic, Ionian and Tyrrhenian Seas, as shown by a Kruskal-Wallis test ($H = 22.87$, $p < 0.001$). A significantly lower median abundance of resting stages was also found in the Ionian samples relative to the Adriatic ones (post-hoc Mann Whitney test with Bonferroni correction, $U = 254$, $p < 0.001$).

While grouping samples according to the basin where they were collected, i.e. according to the most natural partition, allowed to point out some general relationships thanks to the RDA,

a more effective partition of the data set was obtained thanks to a Multivariate Regression Tree, which was mainly driven by SST and depth and only partly reflected subregional differences between basins. In fact, the MRT allowed to find the most parsimonious optimal partition of the resting stage data set, as well as the environmental variables and the cut-off values that defined that partition. As it was based on Euclidean distances computed from log-transformed abundances, it operated in the same data space as the RDA.

The analysis of the cross-validation error based on 100 iterations (Fig. S2) showed that the minimum value (0.5998) was obtained for a MRT with three leaves. This was also the most parsimonious tree within one standard error from the minimum value of the cross-validation error and therefore it was selected as the optimal solution also according to Breiman et al. (1984) (Fig. 4). The first split was based on the average SST, with a cut-off value of 18.57 °C, and generated a first leaf, including 26 sediment samples from cooler waters, and second split was based on depth. The latter split was associated to a cut-off value of 368 m and the two leaves that were generated included 53 shallower sediment samples and 15 deeper ones, respectively. Leaves in the MRT were labelled with symbols (gray circle, black triangle and white square) which have been consistently used as reference.

While the average within-group Euclidean distance was 6.303 when samples were grouped by subregional sea, the groups obtained from the MRT leaves were more homogenous, with a 5.050 average within-group Euclidean distance. The difference was highly significant ($t = 15.047$, $p << 0.001$) and showed that the partition generated by the MRT fitted the resting stage data much better than the one based on a strictly geographical criterion, although the latter can be regarded as a proxy for several relevant ecological and oceanographic features.

Each leaf in the MRT was associated to the average composition of the subset of resting stage samples it represents as shown in the small bar diagrams. While the three average assemblages clearly differed from each other, they did not allow an easy comparison of their

composition and the only feature that could be easily grasped was the major role played by the taxa in the first two bars from the left, i.e. dinoflagellate cysts and diatom spores, respectively. These taxa were dominant in all the leaves, although to a lesser extent in the leftmost one, including 15 sediment samples. A more complete analysis of the average resting stage assemblages obtained from the MRT was carried out by means of a SIMPER procedure (Table 1). The various taxa were ranked according to their contribution to the average Euclidean distance between the resting stage assemblages associated to the three leaves of the MRT. Distances were computed, as in the previous analyses, based on $\log_{10}(x+1)$ transformed abundances (cysts or spores g^{-1} d.w.). For each taxon, from left to right, the average distance between the groups of samples in the MRT leaves, the percent contribution to the overall average distance and the cumulative percent contribution to the overall distance were shown. The log-transformed average abundance in the assemblages associated to each leaf, were represented as a horizontal bar and as a number. The three rightmost columns, showing the average structure of the resting stage assemblages, can be associated to the corresponding leaf in the MRT by means of the symbol or of the cut-off values for temperature (SST) and depth.

As previously mentioned, the two broadest taxa (dinoflagellate cysts and diatom spores) played important roles, but also two species provided comparable contributions to the overall distance between resting stage assemblages, such as *A. minutum* cysts and *D. brightwellii* spores. Both species contributed more than 20% of the overall distance and together with the two broadest taxa accounted for more than 50% of the overall distance between the samples. Six more taxa, at species or genus level, accounted for an additional 45% of the overall distance, thus leaving less than 5% of the overall distance to the seven least abundant taxa.

The general pattern that emerged from the MRT and SIMPER analysis of its leaves showed that the average abundance of resting stages was maximum in samples collected at sites with

average SST lower than 18.57 °C, while intermediate abundances were found at warmer stations whose depth was less than 368 m. Deeper stations whose average SST was higher than 18.57 °C had the poorest resting stage assemblages, although dinoflagellate cysts and diatom spores still played the most important role. The abundance of all taxa was monotonically decreasing from right to left in the MRT and from left to right in Table 1, with the only exception of the *C. socialis* spores, which are slightly more abundant in the deepest subset of warmer samples than in the shallower ones.

In the Mediterranean Sea, the spatial distribution of the sediment samples was assigned to each leaf of the MRT (Fig. 5). Different shades of gray show the distribution of the variables, which were selected for the MRT splits, as average SST and depth. Marine regions in white were those where the average SST was lower than 18.57 °C, and included northern and Central Adriatic Sea in addition to the western Ligurian Sea, the northern western Mediterranean and two regions eastern and western of the Strait of Bonifacio where the effects of the mistral frequently cool down the upper layer of the water column. However, no sediment samples were available in the latter regions, while several samples (n = 26) were collected at shallow sites with average SST values below 18.57 °C in coastal Adriatic regions at North of 41°, and all of them belonged to the first leaf of the MRT. This grouping included the highest abundances of resting stages were associated. The average depth of this group of sampling stations was 36 m. All the remaining samples belonged to one of the two leaves generated by the second split in the MRT, which was based on depth, with 368 m as cut-off value. The shallower region was shown in light gray and included 53 sampling stations, scattered along the Ionian and Tyrrhenian neritic waters. In few cases, water depth was down to the upper part of the continental slope, but with an average depth of 130 m. The deepest stations (n = 15), with an average depth of 641 m, were located in most cases very close to

the boundary between the shallower waters and the deeper region that stretches beyond the 368 m isobaths, down to the deepest part of the Ionian and Tyrrhenian basins.

4. DISCUSSION

In the present study, target phytoplankton taxa producing resting stages were studied based on their higher presence and abundance, and also for their bloom formation in the Mediterranean seed banks and surface water, respectively. Among phytoplankton species forming resting stages, target HAB taxa were also investigated as they could represent a threatening for new blooms origination especially at coastal areas. These species are known to be widespread and abundant in the Mediterranean sampled areas (Rubino et al. 2009, Anglés et al. 2010, Satta et al. 2010, Penna et al. 2010, Montresor et al. 2013, Piredda et al. 2017, Perini et al. 2019).

Due to the huge extension of the Mediterranean bottom, the number of known and unknown taxa, and the relative amount of molecular primers to be used in qPCR assay, it is not yet feasible to quantify all diversity of resting stage taxa present in the sediments. As far to date, few molecular data are present to inform us about the diversity of resting stages produced by diatoms or dinoflagellates in the Mediterranean Sea (Piredda et al. 2017, Perini et al. 2019). So, it is challenge starting to explore the diversity of resting stage assemblages in this basin in order to know taxa richness and structure of resting stage assemblages in relation to environmental variables and human impact. Regional seas were sampled at coastal and off-shore areas in the Mediterranean basin. The molecular analysis of target resting stage of phytoplankton assemblages in marine sediments of the Central Mediterranean Sea was carried out by using more sensitive and specific qPCR assay than traditional microscopy.

Among the molecular quantified resting stages, presumably non-viable dormant stages of *Pseudo-nitzschia* were found in the majority of samples. However, it is still known that

formation of resting stages by *Pseudo-nitzschia* has not been reported (Piredda et al. 2017). However, it was hypothesized that *Pseudo-nitzschia* spp. may have a quiescent phase either in the water column or in the sediment, during which cell division, and therefore size reduction, are markedly reduced (Amato et al. 2005). In support of this hypothesis, some authors documented the presence of *Pseudo-nitzschia* spp. preserved valves in sediments (Parsons & Dortch 2002, Abrantes et al. 2007, Lundholm et al. 2010). In particular, preserved valves of *Pseudo-nitzschia* spp. and intact cells of *P. pungens* with cell content in the core sediments were found and presumably these could be non-viable dormant stages.

The molecular approach has been successfully applied mainly for the quantification of target harmful microalgal species in different environmental matrices allowing a rapid examinations of field samples. High-throughput qPCR platforms permit the simultaneous processing of a large number of samples, significantly decreasing processing time compared to microscopic procedures that also require high level of taxonomical expertise (Fitzpatrick et al. 2010, Perini et al. 2011, Casabianca et al. 2013, Penna et al. 2013, Casabianca et al. 2014, Kon et al. 2017).

In the present study, the decreasing of resting stage abundances of dinoflagellates and diatoms, which are considered the two dominant groups in marine phytoplankton, in relation to depth down to about 1000 m was a clear evidence emerging from statistical analyses. Resting stage abundance was found to be higher in shallower sites than in deeper ones, with larger amounts of resting stages produced by diatoms rather than by dinoflagellates. Resting stages have different sinking rates in the water column based on their peculiar biological and physical properties. Dinoflagellate cysts and diatom spores retain sinking rates between 6 – 11 m d^{-1} and 0.57–16 m d^{-1}, respectively (Anderson et al. 1985, French & Hargraves 1980). However, resting spores can also aggregate passively in marine snow with a sinking rate of 43 – 95 m d^{-1} (Shanks & Trent 1980), which is faster than that of individual resting stages.

Therefore, cysts and spores reach the sea floor with different sinking times. In the present study, we assessed that spores and cysts likely sink down to the deepest station (1018 m) in a lapse of time ranging between 15 days and 4 months.

Furthermore, seawater trophic conditions can influence the structure of phytoplankton assemblages in term of species abundance, composition and succession (Hernández-Fariñas et al. 2014, Otero et al. 2018) and related formation of resting stages. Major phytoplankton groups often differ in average functional trait values, thus implying diverse performance and fitness under environmental conditions (Barton et al. 2013). Diatoms, often of small size, seem to follow the r-strategy in mixing waters characterized by high nutrient concentrations. They are typically dominant in coastal waters that receive riverine input as well as open waters in favourable upwelling conditions. Instead, dinoflagellates are expected to be more abundant in calm, confined and warm coastal waters (Finkel et al. 2010, Mutshinda et al. 2016). These trophic and hydrodynamic conditions are found in the Mediterranean areas where sampling activities were carried out (Valbi et al. 2019). Resting diatom spore abundance was higher than dinoflagellate cyst amount in all sampled areas with the highest abundance in the Adriatic Sea. This latter aspect could be related to the mesotrophic conditions, especially in the north and central portion of the basin, where it receives major riverine input. This environment is often favourable for diatom blooms which can sustain encystment processes in the water column. Further, high spore density was found below 20 m reaching the highest values (10 - 27 x 10^4 cysts g^{-1} d.w.) from 31 to 65 m. The diatom *D. brightwellii* was the most abundant species followed by *C. socialis* and *Thalassiosira* spp. In the other two basins considered, i.e. Ionian and Tyrrhenian, spores were found in lower abundances, although in deeper stations in comparison with Adriatic Sea. In general, dinoflagellate cysts were found in lower abundance in all sampled areas (below 10^4 cysts g^{-1} d.w.) and highest amounts were found below 30 m. Again, the Adriatic Sea resulted the most

productive area for cyst deposition on surface bottom. Among the dinoflagellate cyst taxa, the potentially harmful species, as *A. minutum* and *Gymnodinium* spp., were found to be the most abundant ones in the range of $10^3 – 10^2$ cysts g^{-1} d.w. The higher abundance of resting stages found mostly below 20 m can be likely associated to a lesser disturbance effect by wave motion or anthropic activities (shipping, harbours, tourism, infrastructures, riverine input and sewage). However, shallower stations presenting higher resting stage abundance provided a seafloor that can be reached very quickly by resting stages and may present more advantageous environmental conditions to their survival (Bastianini et al. 2016).

Multivariate data analyses confirmed that the abundance of the resting stage assemblages seemed related to depth, as it was consistently higher in shallower samples. While this general pattern is very clear even when single taxa were considered, differences in assemblage structure showed that depth was not the only variable affecting distribution and abundance of the resting stages.

The ordination obtained from the RDA, with almost 50% of the total variance explained by the first two axes, showed regional differences in the composition of the resting stage assemblages, but it also showed how environmental drivers were related with those regional differences. Permutation-based test confirmed that this overall relationship as well as the two above-mentioned canonical axes were significantly different from those expected by chance and therefore, the dependence of the resting stage assemblages from the environmental conditions can be regarded as something more than a simple hypothesis.

However, while RDA allowed analyzing the relationships between the resting stages and explanatory environmental variables, the latter were not independent from each other and not all of them were necessarily relevant to the biotic response. As a consequence, RDA depicted a general pattern that was not independent of environmental drivers, but it did not allow to point out the most relevant among the latter.

The partition of the resting stage assemblages obtained from the MRT and the environmental variables that were selected to define its splits provided additional insights that were consistent with the results of the RDA. Interpreting the role of the environmental variables that were selected at MRT as causal was certainly tempting, but such an assumption could prove to be misleading. In fact, environmental variables played a role that was only based on similarities between their spatial distribution and the spatial distribution of the target, which in the case of this MRT was the overall structure of the resting stage assemblages, as described by Euclidean distances between log-transformed lists of taxa. In practice, when a MRT is trained, a variable is selected at a given split if the "shape" of a suitable threshold in its spatial distribution matches the best partition of the biotic data (sub)set.

Although average SST and depth may not be causally linked to the structure of the resting stage assemblages, the partition they defined was actually more effective than the one based on geographical distribution only. As for average SST, it probably does not play a direct role in the accumulation of resting stages on the sea floor, but it certainly makes sampling stations in North and Central Adriatic Sea different from all others, with the only exception of a slightly warmer region roughly corresponding to the Mid Adriatic Pit, which unfortunately was not sampled. However, North and Central Adriatic waters are not only cooler, but also very shallow and under the effects of intense terrestrial inputs, as river discharge accounts for an average input of 2500 m^3 s^{-1} of fresh water, i.e. more than 30% of the overall river discharge in the Mediterranean Sea (Struglia et al. 2004).

While a small difference in average SST can hardly be a sufficient cause for large scale ecological effects, extreme events, which are not rare in shallow coastal waters under the effects of point and diffuse terrestrial run-off, may have occasionally played such a causal role. As a matter of fact, the SST range in Central and especially in Northern Adriatic Sea is the second widest in the Mediterranean Sea, after the Black Sea (Shaltout & Omstedt 2014),

and extreme events, with SST well below 10 °C and sometimes as low as 7-8 °C are not rare in coastal regions. As for depth, which was the second explanatory variable selected by the MRT, causality in driving the composition of resting stage assemblages can be reasonably excluded. Nevertheless, depth it is not independent of distance from the coastline, which in turn is related, for instance, to nutrient availability and phytoplankton biomass, which may have played a role in the accumulation of phytoplankton resting stages. While the latter explanatory variables were available to the MRT, they were not selected, and this obviously means that their effects were mediated by more complex relationships, which remain to be explored.

However, the partition obtained from the MRT was significantly more effective than the one based on a strictly geographical criterion, thus showing that average SST and depth played a role, independently of the causal or correlational nature of the relationships that link them to the resting stage assemblages. As we opted for the most parsimonious MRT, only two explanatory variables were selected. In case more resting stage samples will become available in the future, a larger data set would most probably allow to train a more complex MRT and to get deeper insights into distribution and structure of phytoplankton resting stage assemblages.

5. CONCLUSIONS

In conclusion, the structure of a resting stage assemblage is obviously not independent of those of all the phytoplankton assemblages that in time and space contributed to its formation. In fact, a resting stage assemblage can be regarded as the time- and space-integrated response of a subset of species to the environmental conditions, including the physical oceanographic dynamics that favored or prevented sedimentation of the resting stages. Therefore, this peculiar diachronic assemblage can be regarded as the integral of the

biotic responses to seasonal changes and to all the other modifications of the environmental conditions of the water column, thus summarizing complex time series. A resting stage assemblage cannot entirely preserve the relevant information in the time series from which it has been generated, but it can be a very valuable target for one-off sampling activities or when water column sampling cannot be repeated with a frequency that is as high as to resolve the complexity of the temporal evolution of phytoplankton assemblages. As sampling of resting stages can be carried out at very low frequency while preserving the possibility of an effective comparison between phytoplankton assemblages, it can be also regarded as a very strong candidate for large scale monitoring of the effects of climate change.

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Table 1. SIMPER analysis of the three assemblages of resting stages defined by the Multivariate Regression Tree (Fig. 4). From left to right: name and short name of the taxon, average Euclidean distance, percentage contribution to the overall Euclidean distance, cumulative percentage contribution to the overall Euclidean distance and average log-transformed abundance in each leaf of the Multivariate Regression Tree (both bars and numerical values are shown).

Taxon	Short name	Average D	% D	Total % D	Temp < 18.57°C	Temp ≥ 18.57° Depth < 368 m	Temp ≥ 18.57° Depth ≥ 368 m
Diatom spores	Diat	3.304	15.610	15.61	3.50	2.84	0.97
Alexandrium minutum cysts	Amin	2.735	12.920	28.53	2.03	0.42	0.00
Ditylum brightwellii spores	Dity	2.433	11.490	40.02	1.64	0.22	0.00
Dinoflagellate cysts	Dint	2.307	10.900	50.92	3.45	2.52	1.25
Skeletonema spp. spores	Skel	1.689	7.981	58.90	1.46	0.08	0.00
Thalassiosira spp. spores	Thal	1.685	7.959	66.86	1.56	0.59	0.10
Protoceratium reticulatum cysts	Prot	1.555	7.348	74.21	1.50	0.46	0.06
Chaetoceros socialis spores	Chae	1.332	6.295	80.50	0.97	0.43	0.47
Gymnodinium spp. cysts	Gymn	1.296	6.122	86.63	1.73	1.09	0.12
Gonyaulax spinifera cysts	Gspi	0.956	4.517	91.14	0.99	0.10	0.10
Pseudo-nitzschia spp. spores	Pseu	0.916	4.325	95.47	1.05	0.67	0.04
Lingulodinium polyedrum cysts	Ling	0.641	3.028	98.50	1.01	0.31	0.10
Scrippsiella trochoidea complex cysts	Scri	0.199	0.938	99.43	0.63	0.28	0.08
Alexandrium tamarense/mediterraneum cysts	Atam	0.091	0.432	99.87	0.21	0.09	0.02
Heterosigma akashiwo cysts	Hets	0.015	0.069	99.94	0.00	0.02	0.00
Alexandrium pacificum cysts	Apac	0.011	0.050	99.99	0.01	0.03	0.00
Heterocapsa triquetra cysts	Hetc	0.003	0.014	100.00	0.00	0.01	0.00

The above values are log $_{10}$ (x+1) cysts/spores g^{-1} DW sediment

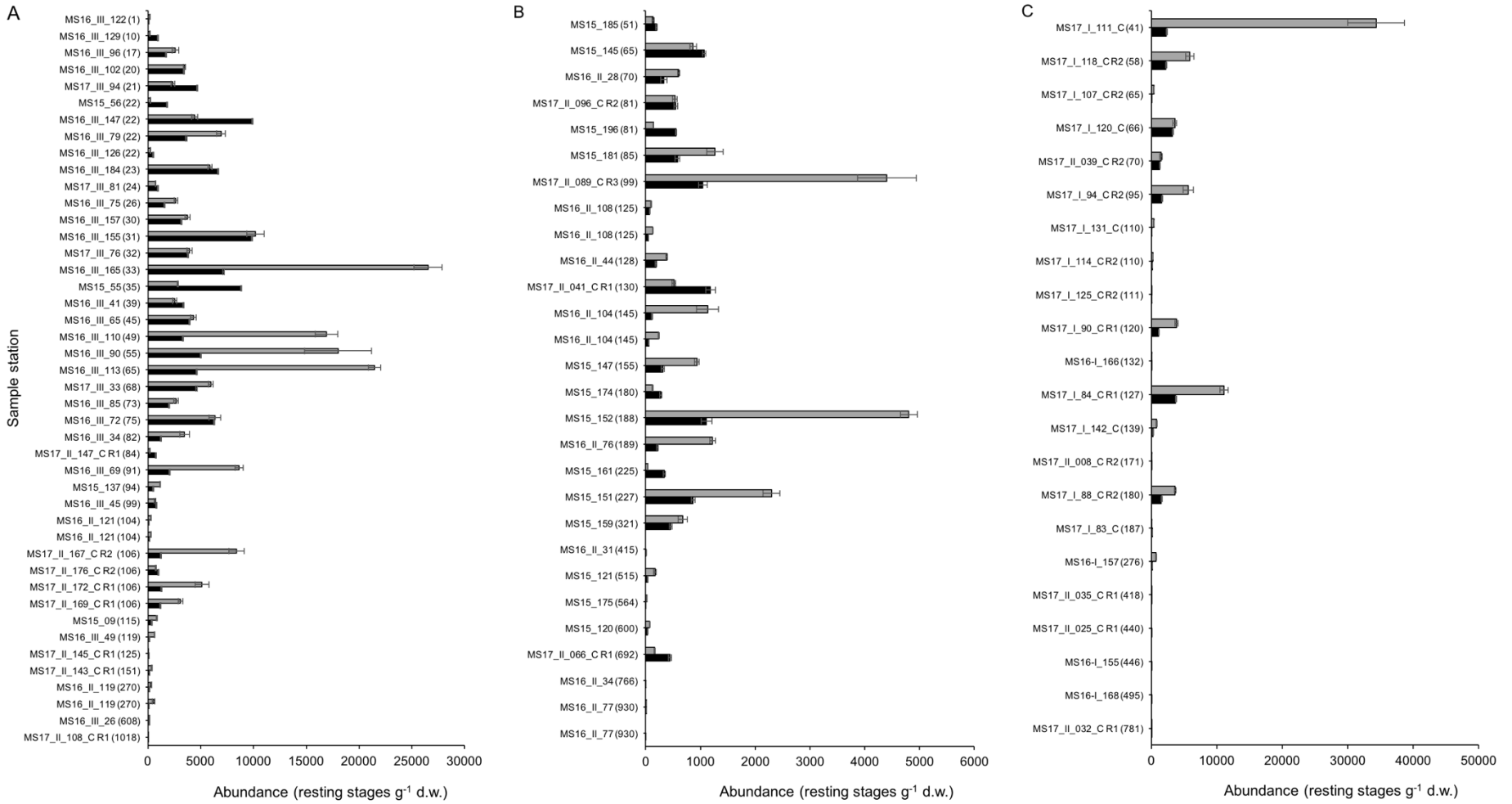


Figure 1. Abundance of resting stages of Dinophyceae (black columns) and Bacillariophyceae (grey columns) expressed as number of resting stages g^{-1} d.w. of sediment determined by qPCR assay in the Mediterranean Sea. Samples on y axis are in order of depth from the less deep to the deepest, the depth, expressed in meters below sea level is reported in brackets. A, Adriatic Sea; B, Ionian Sea; C, Tyrrhenian Sea.

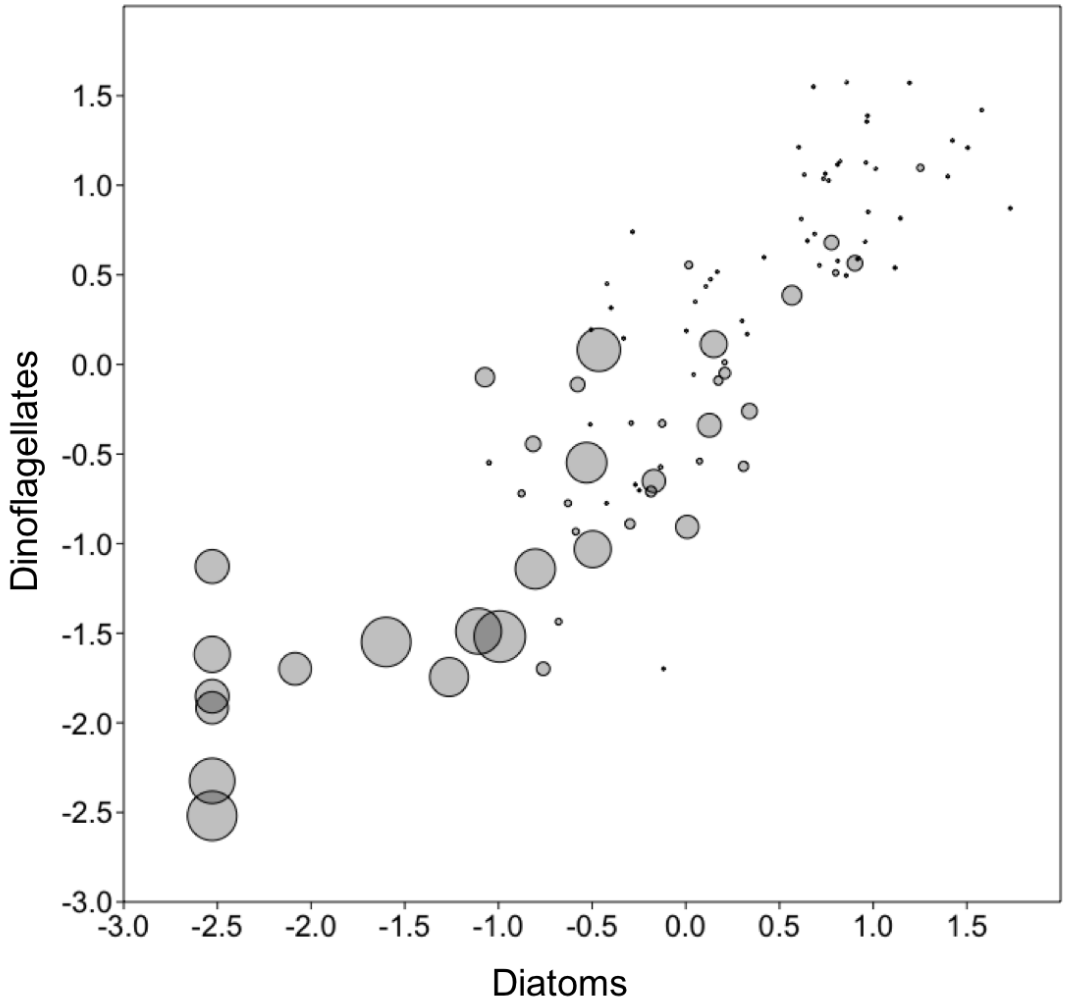


Figure 2. Total abundance of dinoflagellates vs. diatoms, expressed as resting stages g^{-1} d.w. of sediment by qPCR based assay. Resting stage abundances were log-transformed as x'= $\log_{10}(x+1)$. Circle size is proportional to depth. While covariation in dinoflagellate and diatom abundances emerges as the main pattern, smaller circles, corresponding to shallower samples, are mostly associated to higher abundances.

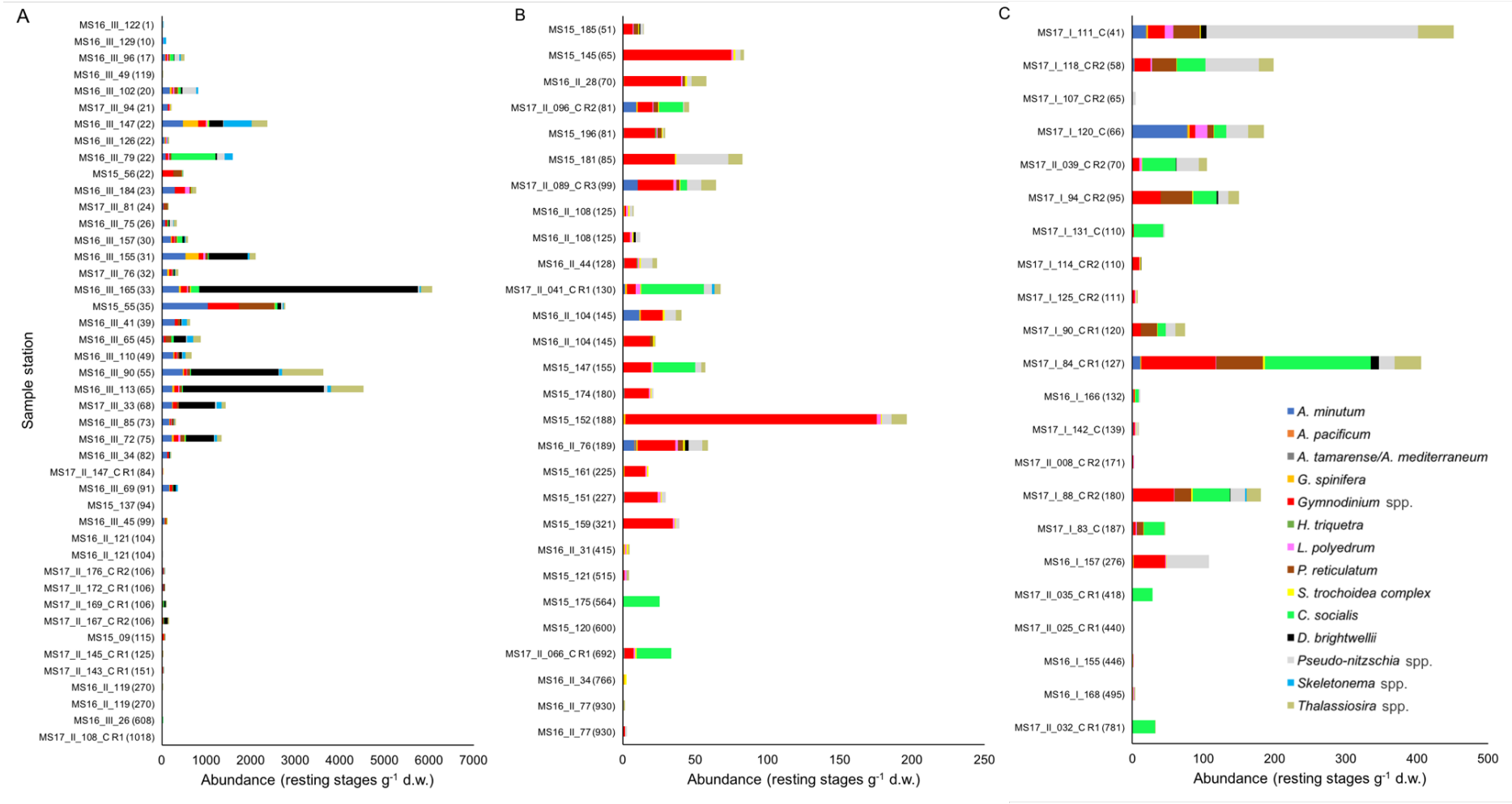


Figure 3. Resting stages abundance expressed as number of resting stages g^{-1} d.w. of sediment determined by qPCR assay in the Mediterranean Sea. Among all resting stage taxa abundance, target toxic producing taxa were also shown (i.e. *A. minutum*, *A. pacificum*, *G. spinifera*, *L.*

1 *polyedrum*, *P. reticulatum*, *Pseudo-nitzschia* spp.). Samples on y axis are in order of depth from the less deep to the deepest, the depth,
2 expressed in meters below sea level is reported in brackets. A, Adriatic Sea; B, Ionian Sea; C, Tyrrhenian Sea.

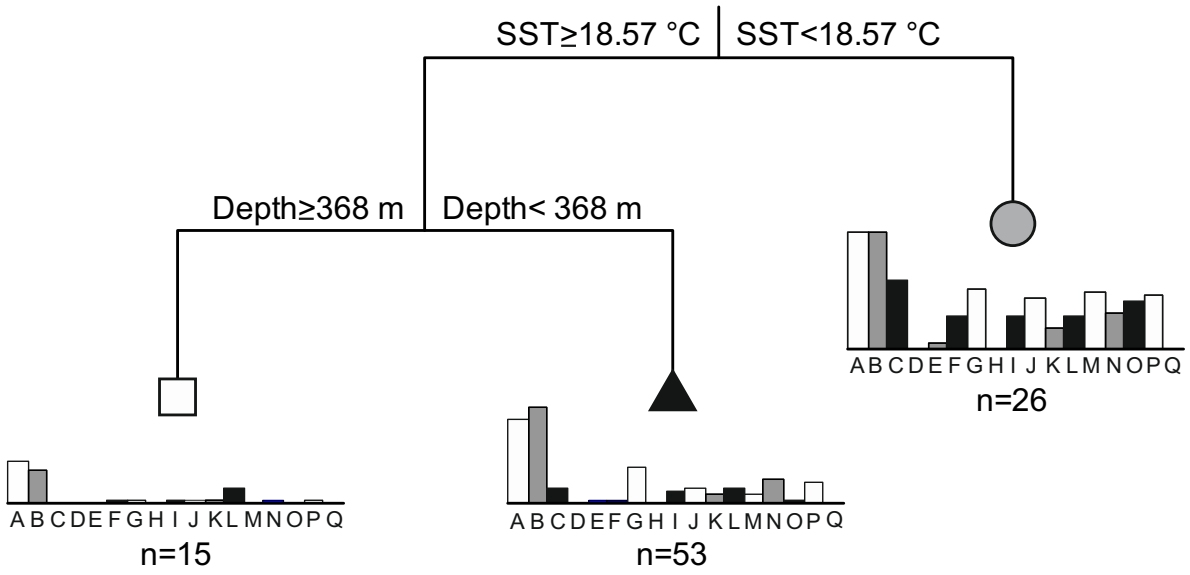
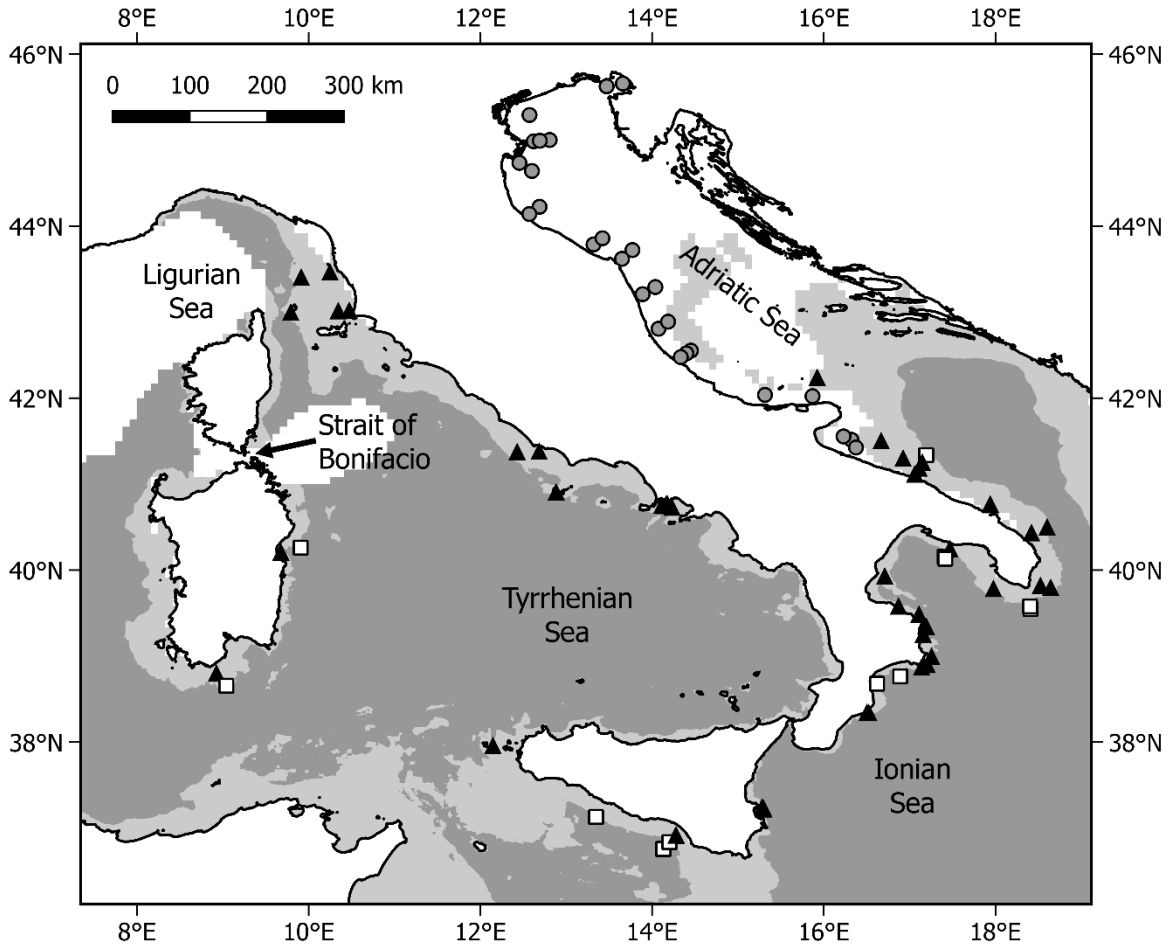


Figure 4. Multivariate Regression Tree. The most parsimonious optimum structure, with three leaves only, was selected according to the cross-validation relative error plot (Fig. S2). The average assemblage structure is shown at each leaf by a bar plot. Abundances are log-transformed and taxa are shown by letters: dinoflagellate cysts (A), diatom spores (B), *A. minutum* cysts (C), *A. pacificum* cysts (D), *A. tamarense/mediterraneum* cysts (E), *G. spinifera* cysts (F), *Gymnodinium* spp. cysts (G), *H. triquetra* cysts (H), *L. polyedrum* cysts (I), *P. reticulatum* cysts (J), *S. trochoidea* complex cysts (K), *C. socialis* spores (L), *D. brightwellii* spores (M), *Pseudo-nitzschia* spp. non-viable dormant stages (N), *Skeletonema* spp. spores (O), *Thalassiosira* spp. spores (P), *H. akashiwo* cysts (Q).

1



2

3 Figure 5. The three different shades of gray show the regions corresponding to the
4 assemblage structures obtained from the MRT. Sampling stations in each region are
5 associated to the same symbols as in Table 1 and Fig. 4. High resting stage densities were
6 found at colder stations in North and Central Adriatic Sea (gray circles on white),
7 intermediate densities at warmer and shallower stations along the coastline of all the other
8 basins (black triangles on light gray) and low density, with reduced species richness, at
9 warmer and deeper stations (white squares on dark gray).